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## Full length article

Atlantic salmon (*Salmo salar*) mounts systemic and mucosal stress responses to peracetic acid

Malene Soleng<sup>a</sup>, Lill-Heidi Johansen<sup>b</sup>, Hanne Johnsen<sup>b</sup>, Gunhild S. Johansson<sup>b</sup>,  
Mette W. Breiland<sup>b</sup>, Lisbeth Rørmark<sup>c</sup>, Karin Pittman<sup>d</sup>, Lars-Flemming Pedersen<sup>e</sup>,  
Carlo C. Lazado<sup>a,\*</sup>

<sup>a</sup> Nofima, Norwegian Institute of Food Fisheries and Aquaculture Research, 1433, Ås, Norway

<sup>b</sup> Nofima, Norwegian Institute of Food Fisheries and Aquaculture Research, 9019, Tromsø, Norway

<sup>c</sup> Lilleborg AS, 0277, Oslo, Norway

<sup>d</sup> Quantidoc AS, 5006, Bergen, Norway

<sup>e</sup> Technical University of Denmark, National Institute of Aquatic Resources, Section for Aquaculture, 9850, Hirtshals, Denmark

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## ABSTRACT

Peracetic acid (PAA), a strong organic peroxide, is considered a relatively sustainable disinfectant in aquaculture because of its broad effectivity against many pathogens at low concentrations and because it degrades spontaneously to harmless residues. The impacts of PAA on fish health must be determined before its use as either a routine disinfectant or chemotherapeutant. Here we investigated the systemic and mucosal stress responses of Atlantic salmon (*Salmo salar*) to PAA. In experiment 1, salmon were exposed to different nominal concentrations (0, 0.6, and 2.4 ppm) of PAA for 5 min, followed by a re-exposure to the same concentrations for 30 min 2 weeks later. Sampling was performed before exposure to PAA and at 2 h, 48 h, and 2 w after exposures. In experiment 2, fish were subjected to crowding stress prior to PAA exposure at 4.8 ppm for 30 min. The fish were sampled before exposure and 1 h, 4 h, and 2 w after. The two trials were performed in a recirculation system. Both systemic (i.e., plasma cortisol, glucose, lactate, total antioxidant capacity) and mucosal (i.e., expression of antioxidant coding genes in the skin and gills) stress indicators were affected by the treatments at varying levels, and it was apparent that the fish were able to mount a robust response to the physiological demands of PAA exposure. The cortisol levels increased in the early hours after exposure and returned to basal level afterwards. Prior exposure history to PAA did not markedly affect the levels of plasma lactate and glucose when fish were re-exposed to PAA. Crowding stress before PAA treatment, however, did alter some of the stress indicators (i.e., lactate, glucose and expression of antioxidant genes in the gills), suggesting that stress history serves as both a confounding and compounding factor on how stress responses to PAA are mobilised. Nonetheless, the changes were not substantial. Gene expression profile analyses revealed that the antioxidant system was more responsive to PAA in the gills than in the skin. The increased antioxidant capacity in the plasma, particularly at 2.4 ppm and higher, indicates that antioxidants were produced to neutralise the internal redox imbalance resulting from PAA exposure. In conclusion, the results show that salmon were able to mount a robust adaptive response to different PAA doses and exposure times, and a combined exposure to stress and PAA. These results underscore the potential of PAA as a chemotherapeutant for salmon at PAA concentrations commonly applied to control parasitic infestations.

## 1. Introduction

Peracetic acid (PAA) is a highly reactive peroxygen compound and is recognised as a sustainable disinfectant in aquaculture [1,2]. PAA is commercially available as an equilibrium mixture of acetic acid, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and water. The potential of PAA for improved

biosecurity in aquaculture is underscored by its broad range of anti-pathogenic activity and rapid decay into neutral residuals (i.e., carbon dioxide, oxygen and water) [1–6]. The fat solubility of PAA significantly contributes to its potent antimicrobial activity [7], in which the main mode of action is oxidative disruption of cell membranes via hydroxyl radicals [8,9]. These radicals interrupt the chemiosmotic

\* Corresponding author.

E-mail address: [carlo.lazado@nofima.no](mailto:carlo.lazado@nofima.no) (C.C. Lazado).

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function of the lipoprotein cytoplasmic membrane and transport [10,11]. Intracellular PAA acts upon essential enzymes, oxidising them, resulting in the impairment of biochemical pathways, active transport across membranes, and intracellular solute levels [12]. It is also suggested that it can oxidise the sensitive sulfhydryl and sulfur bonds in proteins, enzymes, and other metabolites [3,8]. These features account for why PAA is potent against a wide range of microorganisms, including *Ichthyophthirius multifiliis*, *Aeromonas salmonicida*, *Flavobacterium columnare*, *Yersinia ruckeri*, *Saprolegnia* spp., *Aphanomyces* spp., and infectious salmon anemia virus [3], where, in most cases, the effective dose is less than  $2 \text{ mg L}^{-1}$  [2].

Most of the studies on the application of PAA in aquaculture have focused on degradation kinetics, antimicrobial activity, impacts on water quality, and biofilter nitrification [2,5,6,13]. Though toxicological data exist [3], the physiological responses of fish to PAA exposure are not well documented, and this might undermine its potential as a sustainable prophylaxis and chemotherapeutant in aquaculture. As a strong oxidant, PAA likely triggers physiological imbalances and hence might require fish to mount suitable countermeasures. The stress axis has been shown to mount an adaptive response to the presence of PAA, and this had been documented in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) [1,14,15]. A hallmark response in these studies demonstrated that PAA-exposed fish exhibited an increase in the levels of plasma cortisol after initial exposure, but repeated exposures to PAA resulted in lower cortisol response. The lower cortisol response indicated that fish might have habituated to PAA, but desensitisation, physiological exhaustion, or PAA-mediated endocrine disruption might also explain the reduced corticosteroid response to repeated PAA treatment. Gesto and colleagues [1] demonstrated that the lower cortisol response after repeated PAA exposure was a true form of habituation since rainbow trout repeatedly exposed to PAA were able to execute a normal physiological stress response when prompted with a secondary stressor. As an oxidant, PAA produces hydroxyl radicals following its decay, and this likely results in an altered redox balance, hence triggering oxidative stress. The antioxidant system acts upon these excess reactive oxygen species (ROS) or radicals, thereby protecting the fish from oxidative damage. How fish mobilise its antioxidants defences to PAA-induced oxidative stress is yet to be demonstrated.

In this study, we explored how the systemic (i.e., plasma) and mucosal (i.e. skin and gills) stress defences are mobilised to counteract the physiological pressures or stressors when Atlantic salmon are exposed to PAA. We measured the classical physiological stress indicators (i.e., cortisol, glucose and lactate) as well as the transcriptional changes of key antioxidant coding genes in mucosal tissues.

## 2. Materials and methods

### 2.1. Ethical statement

All fish handling procedures complied with the Guidelines of the European Union (2010/63/UE), as well as with national legislation.

### 2.2. Experimental fish and husbandry conditions

Salmon smolts (Experiment 1:  $150.3 \pm 5.6 \text{ g}$ , mean  $\pm$  SE; Experiment 2:  $131.3 \pm 2.3 \text{ g}$ ) were purchased from Danish Salmon A/S (Hirtshals, Denmark). **Experiment 1:** Upon arrival at the recirculation aquaculture (RAS) facility of DTU Aqua (Hirtshals, DK), fish were sorted and moved to six  $1\text{-m}^2$  holding tanks (water volume  $\approx 600 \text{ L}$ ), with 60 fish in each tank. The RAS had a  $40\text{-}\mu\text{m}$  drum filter, a submerged fixed bed biofilter, and a trickling filter with a makeup water exchange at approximately  $0.4 \text{ m}^3/\text{h}$ , equivalent to a retention time of 1.5 days. Internal recirculation allowed more than two-times the tank exchange per hour. Fish were acclimated for 3 weeks under stable conditions, with daily monitoring of water quality parameters, which

were kept within safe limits (Supplementary Table 1). The tanks had no direct light above them, and the photoperiod in the experimental hall was set at 16L:8D (06.00–22.00), similar to the natural photoperiod in April–May 2018 ( $57^\circ 35' \text{N}$   $09^\circ 57' \text{E}$ ). Water temperature was at  $15 \pm 1^\circ \text{C}$ . The fish were fed (Biomar, EFICO Enviro, 4,5 mm) at a ratio of 1.0–1.5% total biomass per day using a belt feeder. Feeding was gradually increased during the acclimation period, and feeding behaviour of the fish in terms of uneaten feed pellets was registered by daily inspection of the swirl separator. **Experiment 2:** A second batch of smolts were transported to the aquaculture facility of DTU Aqua, sorted and moved to two  $4\text{-m}^2$  holding tanks (water volume  $\approx 1500 \text{ L}$ ) in a seawater flow-through system, with approximately 100 fish in each tank. The fish acclimated for 2 weeks under stable rearing conditions, with daily monitoring of water quality parameters (Supplementary Table 2). Water temperature was at  $11 \pm 1^\circ \text{C}$ . The photoperiod was set at 24L:0D and the dietary ration of 1–1.5% total biomass (Biomar, EFICO Enviro, 4,5 mm) per day was provided using a belt feeder.

### 2.3. Peracetic acid exposure experiments

Peracetic acid (Divosan Forte™, PAA) was supplied by Lilleborg AS (Oslo, Norway). The disinfectant is a stabilised PAA solution (15% v/v) which is non-foaming and completely free-rinsing. The actual amount of PAA in the solution was verified by the DTU Aqua laboratory (Hirtshals, Denmark) to be at approximately  $\sim 18\% \text{ v/v}$ . The solution was stored at  $4^\circ \text{C}$ . During each exposure, the concentration of PAA in the water was experimentally verified [5] in real-time to ensure that the fish were exposed to the target concentration from start to termination of exposure.

**Experiment 1:** To represent the pre-exposure fish, on the day before the first exposure, two fish from each of the holding tanks were sampled, as described in detail in Section 2.4. Feeding was temporarily ceased 24 h prior to PAA exposure. Fish were netted from the holding tank, transferred to a transportation container, and immediately thereafter into a 300-L exposure tank. Each holding tank had its equivalent exposure tank, and water quality parameters were identical between these two tanks. The fish were allowed to settle for 10 min before the PAA solution was added to the tanks to achieve the following final concentrations: 0 (seawater), 0.6, and 2.4 ppm. Even PAA distribution was assured by vigorous aeration directly into the rearing tanks. The concentrations were pre-selected based on an earlier report on the toxicity of PAA for rainbow trout [3]. Each treatment group had two replicate tanks. During the exposure period, the water flow to the tanks was stopped, and the decay of PAA in the water matrix was followed. After 5 min, fish were immediately netted out of the tank and returned to their corresponding holding tank. Post-exposure samplings were carried out thereafter, as detailed in Section 2.4. Two days after the PAA exposure, feeding was resumed, similar to the protocol in Section 2.2. All husbandry conditions during post-exposure rearing were similar to pre-exposure conditions. Two weeks after the first exposure, the fish were re-exposed to the same concentration of PAA. The protocol used in the re-exposure experiment was identical with the approach employed in the initial exposure, with a slight modification on the duration of exposure. Instead of 5 min, fish were re-exposed to PAA at a similar concentration used in the first trial for 30 min. Fish were returned to their corresponding recovery tank, and post-exposure samplings were carried out thereafter. Post-exposure husbandry strategies, as described in Section 2.2, were followed.

**Experiment 2:** Fish were starved for 24 h prior to the exposure experiment. Before the experiment was carried out, four fish were collected from each holding tank to represent the pre-exposure fish. Fish were transferred to a closed-system 500 L exposure tank to achieve a density of  $15 \text{ kg/m}^3$ . The fish were allowed to settle for 15 min before a group was subjected to crowding stress for 1 h, by lowering the water volume to attain a density of  $75 \text{ kg/m}^3$ . Aeration was provided during crowding stress. Fifteen minutes after the water level returned to the

**Table 1**  
Primers used in the present study.

Gene name	Abbreviation	Sequence (5′-3′)	Reference
<i>glutathione peroxidase</i>	<i>gpx</i>	F: GATTTCGTTCCAAACTTCTCTGCTA R: GCTCCAGAACAGCCTGTITG	[35]
<i>glutathione reductase</i>	<i>gr</i>	F: CCAGTGATGGCTTTTGAACCTT R: CCGGCCCCCACTATGAC	[35]
<i>manganese superoxide dismutase</i>	<i>mnsod</i>	F: GTTTCCTCTCCAGCCTGCTCTAAG R: CCGCTCTCCTTGTGGAAGC	[35]
<i>copper/zinc superoxide dismutase</i>	<i>cu/znsod</i>	F: CCACGTCCATGCCTTTGG R: TCAGCTGCTGACAGTCACGTT	[35]
<i>β-actin</i>	<i>β-actin</i>	F: CAGCCCTCCTTCTCGGTAT R: CGTCACACTTCATGATGGAGTTG	[37]
<i>18s</i>	<i>18S</i>	F: TGTGCCGCTAGAGGTGAAATT R: GCAAATGCTTTCGCTTTCG	[38]
<i>elongation factor 1 alpha</i>	<i>ef1a</i>	F: CGCCAACATGGGCTGG R: TCACACCATTGGCGTTACCA	[37]

initial level, one group of the stressed fish was exposed to 4.8 ppm PAA, double the highest concentration tested in experiment 1, while the other stressed group was exposed to 0 ppm (seawater) for 30 min. Likewise, another group of fish was transferred to the same exposure tank but was not exposed to crowding stress. After allowing the fish to settle for 15 min, one group was exposed to 4.8 ppm PAA, and one group was exposed to 0 ppm (seawater) PAA for 30 min. After the exposure experiment, fish were transferred to their corresponding recovery tank, similar to what was used in experiment 1. Post-treatment husbandry protocols were followed, as detailed in Section 2.2. Each treatment group was represented with duplicate tanks.

#### 2.4. Sample collection

For experiment 1, sampling was conducted at 2 h, 48 h and 2 w after exposure for each occasion. For experiment 2, sampling was carried out at 1 h, 4 h, and 2 w after PAA exposure. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of 20% benzocaine solution. After the length and weight were measured, blood was withdrawn from the caudal artery using a heparinised vacutainer, centrifuged at  $1000 \times g$  for 10 min at 4 °C, and plasma was collected and kept at –80 °C until analyses. The same sampling protocol was applied for fish that were collected before exposure. Tissue samples were collected for RNA isolation. A portion of the dorsal skin and the second gill arch was dissected and transferred to RNAlater (Ambion, USA). Tissue samples in RNAlater were left at room temperature overnight and thereafter kept at –80 °C before RNA extraction.

#### 2.5. Plasma stress indicators

Three commercially available assay kits were used to evaluate the level of plasma stress indicators (cortisol, glucose, and lactate). Plasma cortisol was analysed using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Neogen, USA), following the manufacturer's protocol. Plasma lactate was analysed using a Lactate Assay Kit (Sigma-Aldrich, USA). Plasma glucose was quantified using a Glucose Assay Kit (Abcam, USA). All samples were run in duplicates.

#### 2.6. Total antioxidant capacity assay

Total antioxidant capacity in the plasma was colourimetrically quantified by a commercial kit, and the level was expressed relative to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E (Sigma-Aldrich).

#### 2.7. Gene expression analysis

Total ribonucleic acid (RNA) was isolated from skin and gills by

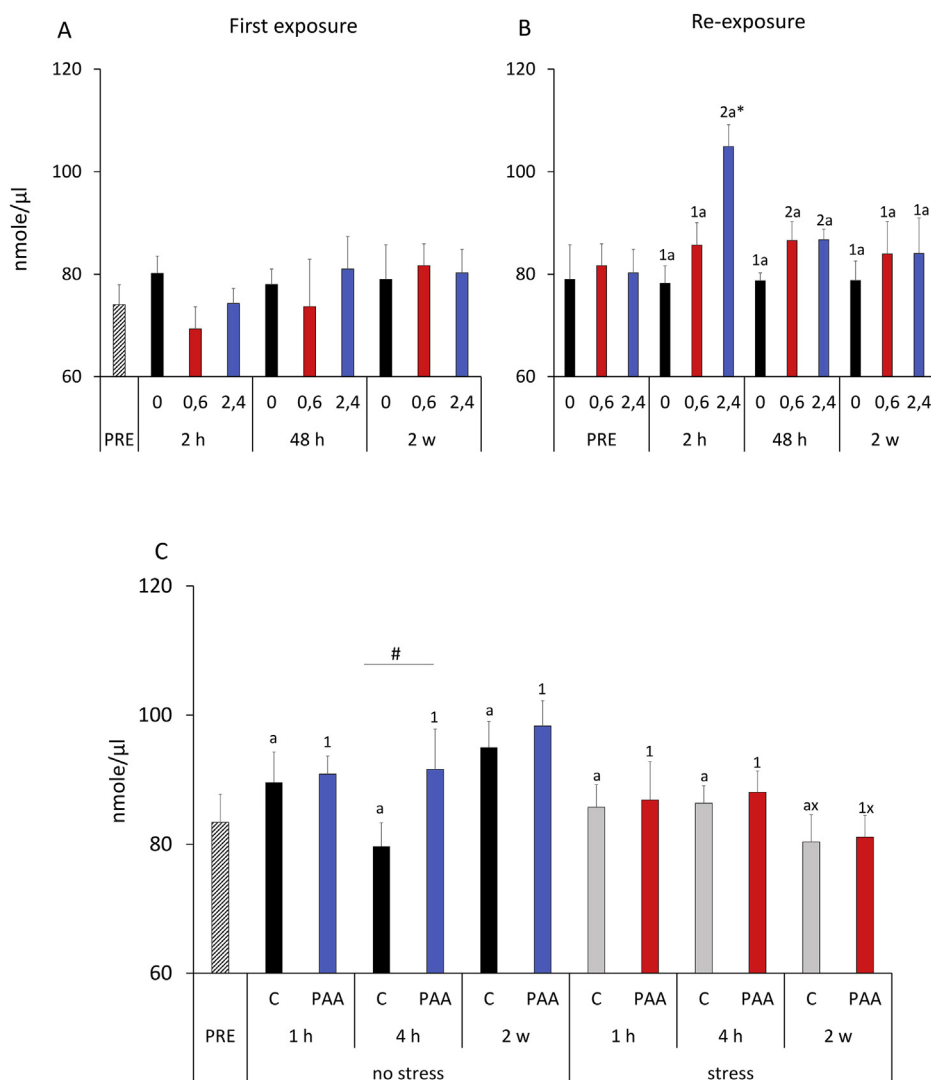
MagMAX TM-96 Total RNA Isolation Kit (Ambion). The RNA concentration and quality were determined using a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). Also, RNA quality was further assessed with an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., Santa Clara, CA, USA). Total RNA was reverse transcribed to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with minor modifications. The 25-μL reaction was set up containing 15 μL (200 ng total) RNA template, 2.5 μL 10X RT Buffer, 1 μL 25X dNTP, 2.5 μL 10X RT random primers, 1.25 μL Multiscript Reverse Transcriptase, 1.75 μL nuclease-free H<sub>2</sub>O, and 1 μL Oligo d(T) (Invitrogen, USA). The thermocycling parameters were as follows: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and 4 °C ∞. Real-time quantitative polymerase chain reaction (qPCR) was performed using the QuantStudio 5 Real-Time PCR system (Applied Biosystems). Each reaction contained 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems), 1.2 μL of each forward/reverse primer (5 μM), 0.6 μL nuclease-free H<sub>2</sub>O, and 7 μL of 1:40 cDNA. Positive and non-template controls (NTC) were included in the assay. The following cycling parameters were used: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. An eight-step standard curve of 2-fold dilution series was prepared from pooled cDNA to calculate the amplification efficiencies. Transcript level was expressed as relative expression following normalisation with the geometric mean of three reference genes (i.e., *β-actin*, *18S ribosomal RNA* and *elongation factor 1 alpha*). The primers used in the study are given in Table 1.

#### 2.8. Statistics

All statistical analyses were performed in Sigmaplot 14.0 Statistical Software (Systat Software Inc., London, UK). A Shapiro-Wilk test was used to evaluate the normal distribution and a Brown-Forsyth test to check for equal variance.

Data sets from experiment 1 were subjected to a two-way ANOVA to test for differences between groups over time. The Holm-Sidak test was used to identify pairwise differences. For experiment 2, a three-way ANOVA was used to test for time, treatments, and stress effects, as well as their interactions. To increase the fit to the model, backward elimination was used to remove insignificant factors from the ANOVA. A Holm-Sidak post-hoc test was applied when significant interactions were detected.

Kruskal-Wallis factor ANOVA and Dunn's post hoc test were used if the requirement for parametric statistics were not met. The transformation was applied where necessary, to meet the assumptions of the two- or three-way ANOVA. If the transformation was unsuccessful, the residuals were plotted for examination. If passed, an ANOVA test was performed. All tests for statistical significance were set at  $P < 0.05$ .



**Fig. 1.** Changes in the level of total antioxidant capacity (TAC) in plasma of fish from Experiment 1 (A & B) and Experiment 2 (C). The level is expressed relative to Trolox standards. Values are mean  $\pm$  SE of eight individual fish. Notations: For graphs A & B (Experiment 1): an asterisk (\*) denotes significant difference between a treatment group and the pre-exposure group (PRE), different numbers signify significant differences between treatments within a sampling point, and different letters indicate significant differences within a treatment through time. For graph C (Experiment 2): different letters denote significant differences within control groups through time, while different numbers indicate differences within PAA exposed groups through time. The same notations are used for no stress and stress groups. x designates that the level of a particular group significantly differs between no stress and stress groups, whereas # indicates a significant difference between the control and PAA-exposed group at a particular time point.

### 3. Results and discussion

The development of any new chemoprophylactic or chemotherapeutic measures in aquaculture should consider the health and welfare consequences for the fish. PAA is widely considered as a sustainable disinfectant in fish farming because of its apparent advantages, yet, little is known how fish respond to this strong oxidant. This is the first report to demonstrate the physiological coping strategies of salmon to oxidative stress induced by PAA. Our results highlight the adaptive responses of salmon to PAA and show that these responses can be altered by either re-exposure or stress history.

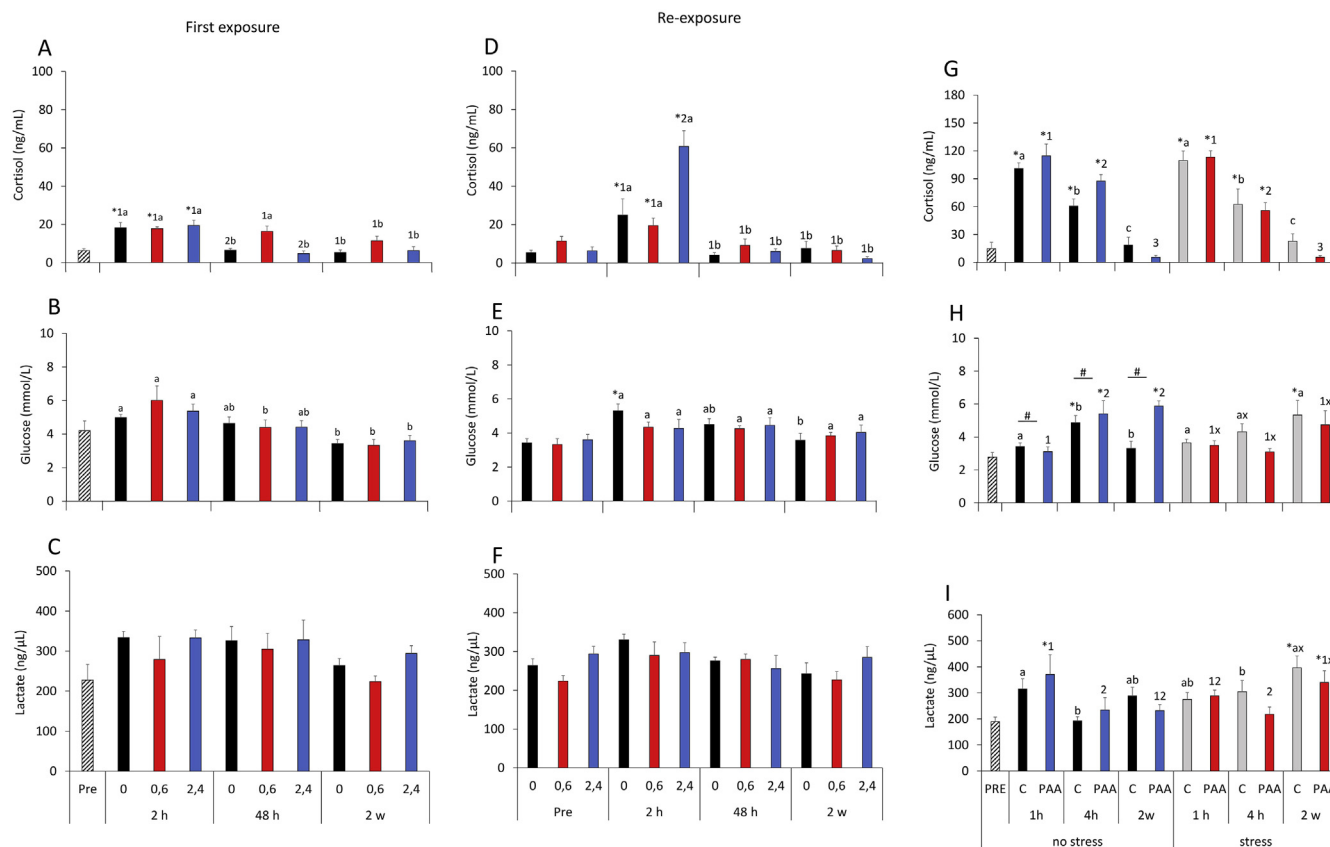
#### 3.1. PAA alters the systemic antioxidant capacity

PAA is a potent oxidant, and its constituents and decay produce forms of hydroxyl radicals and other reactive oxygen species [8,9]. Several studies have shown that the increase in the total antioxidant capacity (TAC) indicates loss of redox balance resulting from oxidative stress, thereby mobilising antioxidants to counteract the alterations [16,17]. Exposing the fish to PAA for 5 min did not trigger significant changes in the TAC of plasma (Fig. 1A). However, when the fish were re-exposed to PAA, but for a longer period, the plasma TAC significantly increased (Fig. 1B). The overall response showed that PAA exposure resulted in increased TAC that lasted for 2 days. At 2 h after exposure, TAC increased by almost 35% in the 2.4 ppm group compared with the

0 ppm group. This remained at a significantly elevated level at 48 h after exposure, though the rate of increase relative to the 0 ppm group decreased to about 10%. There was a lag response in the 0.6 ppm group as TAC significantly increased, but only after 48 h, and at a similar rate of change as with 2.4 ppm when compared with the 0 ppm. No inter-treatment differences were observed at 2 w after the re-exposure, indicating that alterations of the antioxidant state were an acute response. Collectively, the increase in plasma TAC indicated that PAA might have triggered oxidative stress, hence the antioxidants were generated to attack the excess reactive oxygen radicals that might otherwise damage lipids, proteins, and DNA.

Crowding stress has been shown to influence the antioxidative state in fish [18]. The second experiment demonstrated that a stressful episode prior to exposure interfered with the systemic antioxidative response to PAA (Fig. 1C). Fish that were not subjected to stress before PAA exposure showed a marked response, notably at 4 h after exposure. In particular, the TAC in fish exposed to PAA increased by 15% compared with the control group, indicating that the fish were able to mobilise their systemic antioxidant repertoire against PAA-induced oxidative stress. Interestingly, the control and PAA-exposed groups that were subjected to crowding stress before PAA exposure displayed no significant differences. These results imply that a stressful episode before PAA exposure might be a confounding factor, and that it restricted the ability of fish to mount systemic antioxidative responses. Moreover, it appeared that regardless of post-stress treatments, crowding stress





**Fig. 2.** Changes in the level of plasma stress indicators (cortisol, glucose and lactate) of fish from Experiment 1 (A–F) and Experiment 2 (G–I). Values are mean  $\pm$  SE of eight individual fish. Please refer to Fig. 1 on statistical notations.

ensued a long-term effect on TAC. Both the control and the PAA-exposed groups of the group subjected to crowding stress had significantly lower TAC at 2 w post-exposure compared with their counterparts in the no stress group at the same time point.

### 3.2. Plasma stress indicators are activated following PAA exposure

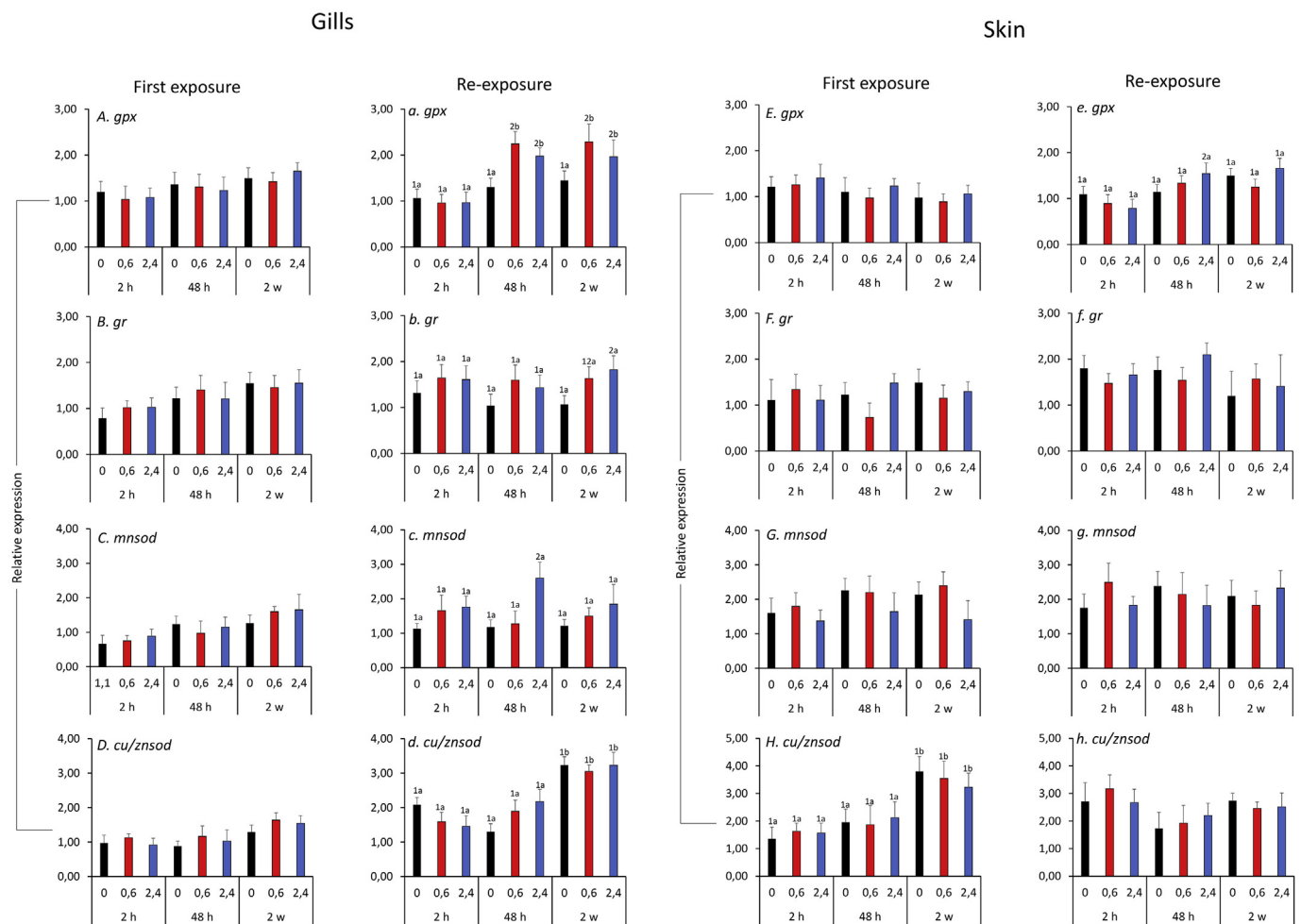
Since the internal redox balance was altered, it was anticipated that stress indicators in the plasma would also change. These interactions might be because of three possible scenarios: 1) the altered redox balance elicits responses from other plasma stress defences to ensure that the organism adapts to the physiological demands of PAA; 2) increased antioxidant activity is a result of altered stress defences; and 3) a simultaneous well-coordinated response from both the classic participants of stress response and antioxidants defence is triggered by PAA. Though the present study could not conclusively identify the mechanisms involved, it is interesting to observe the changes in the plasma parameters during PAA exposure following crowding stress.

Plasma cortisol levels from experiments 1 and 2 followed the same pattern (Fig. 2A) - a significant increase in the early hours after stress had been triggered, then followed by a decrease and return to the baseline values thereafter, which is the classical cortisol response to stress in many teleost fish [19,20]. Moreover, this was in line with other studies on stress response in salmonids, including experiments on peroxide exposure [1,21–23]. In the first exposure in experiment 1, all groups showed significantly elevated cortisol level 2 h after exposure, but inter-treatment differences were not observed. This suggests that the increase in cortisol response might be due to handling during the transfer of fish from the holding/recovery tank to the exposure tank, and not due to PAA. The elevated cortisol level of the 0.6 ppm group after 48 h was striking. Though it is quite difficult to provide a firm conclusion with the current data, we can deduce that 0.6 ppm PAA

combined with handling-related stress has a more acute impact than exposing the fish to 2.4 ppm. This marked difference was not observed when the fish were re-exposed to PAA.

A distinctive rise in cortisol level was observed in the 2.4 ppm group at 2 h post-re-exposure (Fig. 2A). Nevertheless, the average plasma cortisol was lower compared with results from other stress studies on salmon [21,23–26], indicating that the fish did experience a stressful episode, but its magnitude was not overly high. The previous history of PAA exposure appeared to magnify the cortisol response only in the 2.4 ppm group. Moreover, the results reveal that the fish recovered rapidly, as all groups had a cortisol level similar to the baseline at 48 h post-re-exposure. Cortisol values in experiment 2 were higher ( $\sim 110$  ng/mL) compared with the values identified in experiment 1, which indicates that the fish experienced a more intense compound stressor (i.e., handling, crowding and PAA) (Fig. 2G). Interestingly, all groups – regardless of treatment (control, PAA, and/or stress) – had identical patterns in their average cortisol response, which was significantly elevated in the first 4 h after exposure. The similarities in the response of the two groups illustrate that potential interactions and additive effects did not alter the ability of fish to mount a cortisol response to a challenging condition.

PAA did not significantly alter the glucose level in either of the exposure occasions in experiment 1, though temporal variability was apparent (Fig. 3B,E). This result reveals that in the tested PAA doses, prior exposure history did not pose a significant impact on glucose metabolism. In experiment 2, however, prior stress history and a higher PAA dose resulted in the differential activation of glucose metabolism (Fig. 2H). At 1 h after exposure, the no stress-control displayed significantly elevated glucose level compared with the no stress-PAA group, though both groups were not significantly different from the baseline value. At 4 h after exposure, plasma glucose of both groups was significantly higher compared with the baseline value and the no stress-



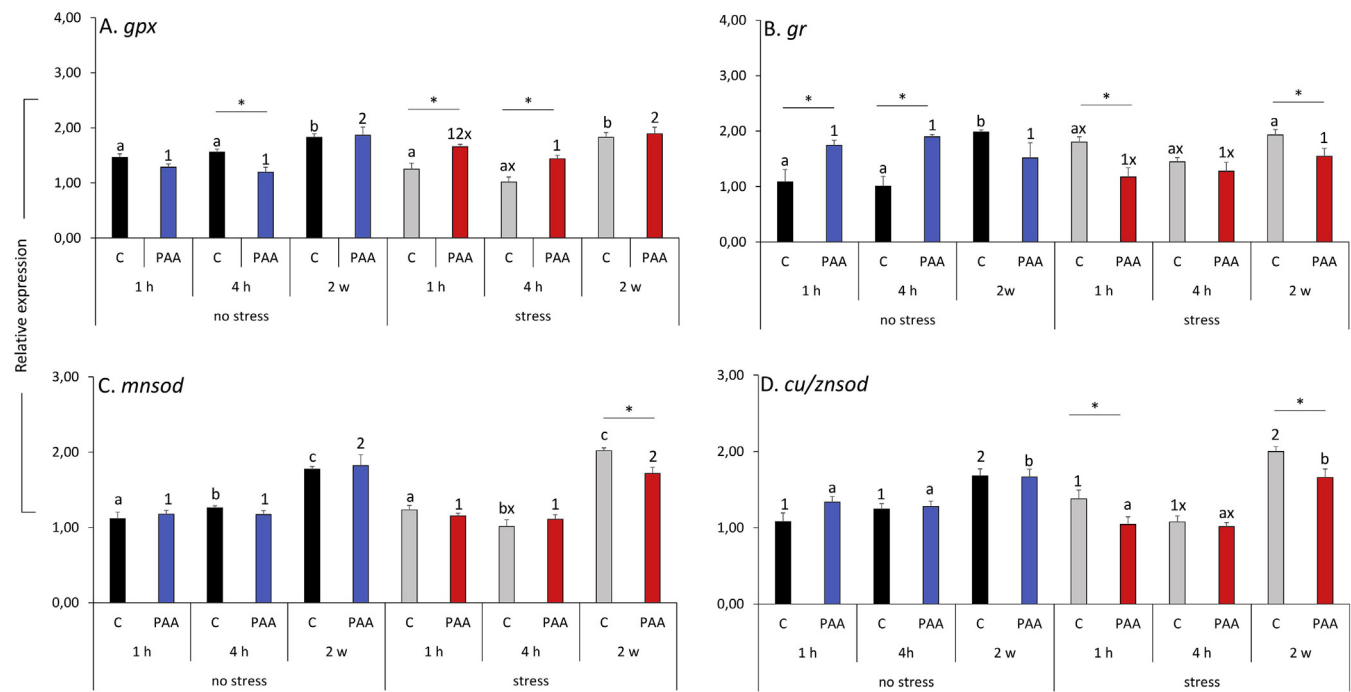
**Fig. 3.** Expression profiles of antioxidant genes in the gills (A,a; B,b; C,c; D,d) and skin (E,e; F,f; G,g; H,h) of salmon from Experiment 1. Values are mean  $\pm$  SE of eight individual fish. Different numbers signify significant differences between treatments within a sampling point, whilst different letters indicate significant differences within a treatment through time. *gpx* = glutathione peroxidase; *gr* = glutathione reductase; *mnsod* = manganese superoxide dismutase; *cu/znsod* = copper/zinc superoxide dismutase.

PAA displayed significantly elevated level compared with the no stress-control. Such an inter-treatment difference was still prominent 2 w post-exposure, though the glucose level of the no stress-control was similar with the baseline value. At this time point, the no stress-PAA group exhibited a glucose level a fold higher than the no stress-control. Prior stress history might interfere with glucose metabolism following PAA exposure (Fig. 2H). No inter-treatment differences were documented in any of the time points, contrary to the profile of the group that was not subjected to a stressful episode prior to PAA exposure. The glucose level 4 h post-exposure was significantly lower for the stress group compared with the no stress group, regardless of the treatment. Moreover, this was still evident 2 w after exposure when comparing the stress-PAA and no stress-PAA groups. Glucose is mobilised following a stressful event to ensure energy is provided to overcome the physiological pressure of the situation [19]. The result in the stress group suggests that the fish might have already mobilised the stored glycogen during the crowding stress [27]. Thus, no adaptive changes were identified when subjected to another stressor. As the glycogen deposit in the liver is limited, no further glucose could be mobilised [28]. This illustrates that crowding stress possibly interferes with the glucose stress response to PAA. The higher glucose level 2 w post-exposure in no stress-PAA indicates a delayed and prolonged effect of the stressors, and that the elevated glucose levels might be due to a heightened state of gluconeogenesis to meet the metabolic demands of PAA and handling [27]. The long-term metabolic consequences of PAA exposure,

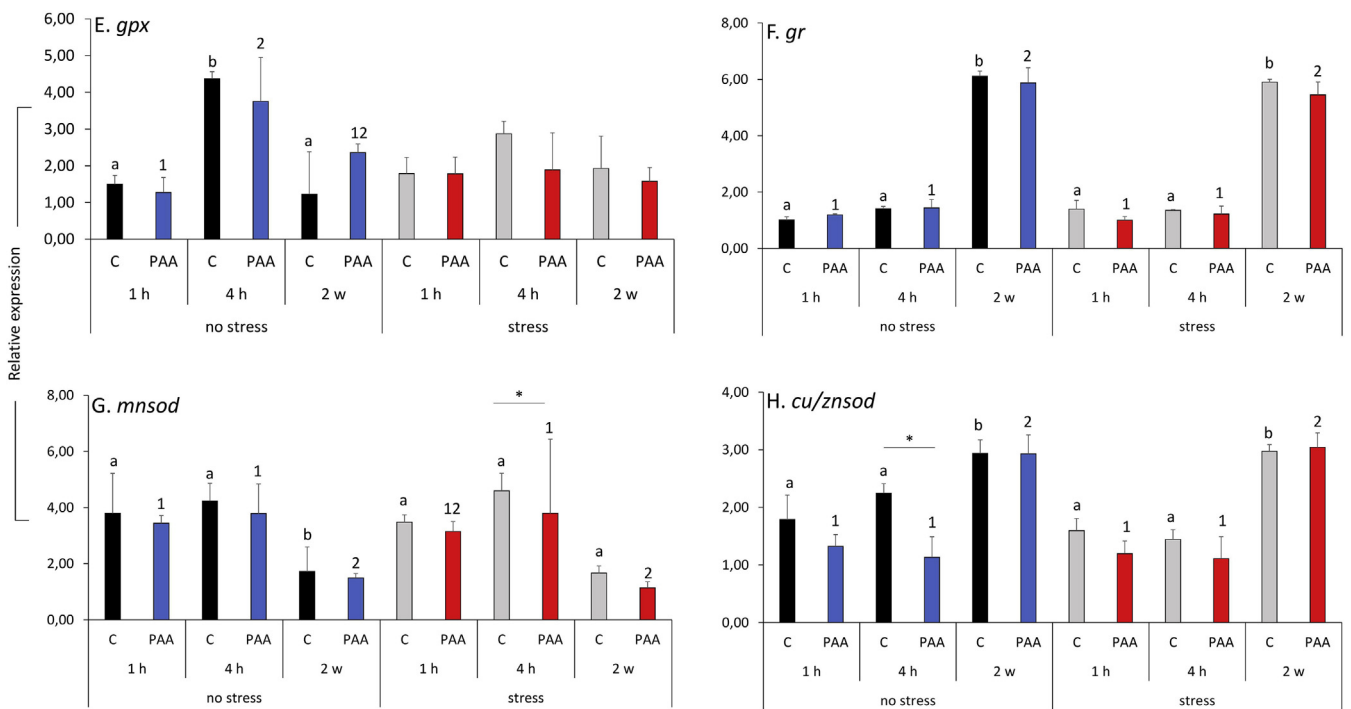
therefore, deserves further investigation.

Lactate is known to increase as a response to a stressful condition [19]. Experiment 1 revealed that the tested PAA concentrations, exposure duration, and re-exposure did not significantly alter the plasma lactate level (Fig. 2C,F). This result corroborates other stress parameters (i.e., cortisol and glucose) in this experiment and further illustrates that though PAA exposure at tested concentrations triggered stress (i.e., changes in plasma cortisol), the magnitude of the stress was not high. The lactate level in experiment 2 revealed more obvious dynamics (Fig. 3D). Plasma lactate of the no stress-PAA group exhibited a significant rise relative to the baseline value 1 h after exposure. The level returned to the basal value thereafter. In addition, no significant difference was identified between no stress-control and no stress-PAA. In contrast to the no stress group, both sub-groups in the stress group had significantly elevated lactate levels 2 w after exposure. We could not ascertain whether crowding stress before exposure might contribute as a compounding factor in the lactate response to an additional stressor (i.e., PAA) since stress-control and stress-PAA displayed no significant difference. It is reported that lactate levels for smolt should not rise above 5 mmol/L ( $\approx$  450 ng/ $\mu$ L) after a stressor [29], and no group had values above this level. The plasma lactate level was slightly higher than the levels found for PAA exposed rainbow trout [1] and on the same level as the control group in a hydrogen peroxide study in salmon [23].

Gills



Skin



**Fig. 4.** Expression profiles of antioxidant genes in the gills (A–D) and skin (E–H) of salmon from Experiment 2. Values are mean  $\pm$  SE of eight individual fish. Different letters denote significant differences within control groups through time, while different numbers indicate differences within PAA exposed groups through time. The same notations are used for no stress and stress groups. x designates that the level of a particular group significantly differs between no stress and stress groups, whereas # indicates a significant difference between the control and PAA-exposed group at a particular time point.



### 3.3. The antioxidant defences are more responsive to PAA in the gills than the skin, and prior stress imposes a potential confounding factor

Oxidative stress occurs when the balance between ROS and the antioxidant defence system is disturbed [30]. Excessive levels of ROS might have detrimental effects on lipid metabolism, protein synthesis, and DNA [31]; therefore, scavenging of the radical surplus must be performed effectively.  $H_2O_2$  and perhaps PAA – since it degrades into  $H_2O_2$  and  $O_2$  – might induce oxidative stress and provoke a defence mechanism against ROS. We have shown that PAA exposure influenced the systemic antioxidant capacity, indicating that oxidative stress might have been triggered during exposure and that a robust humoral antioxidant defence was mounted (Fig. 1). We then asked whether such an antioxidative defence could also be elicited from the mucosal surfaces since they are in contact with water to which PAA was added. The gills and skin are mucosal tissues that function as the first line of defence and are highly responsive to changes in the immediate environment [32], including the levels of ROS. In experiment 1, initial PAA exposure did not affect the expression of any of the antioxidant genes in both tissues, though time-dependent changes in some of the treatment groups were observed (Fig. 3). Re-exposing the fish to the same PAA concentrations resulted in the differential modulation of antioxidant gene expression, particularly in the gills (Fig. 3a,b,c,d). The expression of *gpx* in the gills of fish subjected to 0.6 and 2.4 ppm was significantly higher compared with the 0 ppm group at 48 h, and the trend persisted until 2 w post-exposure (Fig. 3a). Upregulated *gpx* expression was only observed in the skin 48 h after exposure (Fig. 3e). *Gpx* was the only studied gene for which expression was modulated both in the skin and gills in experiment 1, implying a critical role for *gpx* in the antioxidant defence against increased ROS at mucosal surfaces, as noted in other fish studies [33,34]. The gill expression of *mnsod* and *gr* was also modulated in the 2.4 ppm group after re-exposure, where the former displayed an earlier response while the latter exhibited a late response. Overall, the not so dramatic changes in the expression of the antioxidant genes in these mucosal tissues suggest that the tested PAA concentrations in experiment 1 were not able to elicit a strong mobilisation of the antioxidant response, which further suggests that the PAA-induced oxidative stress was not strong at the mucosa.

The gene expression profile in experiment 2 corroborated the results in experiment 1; that the antioxidant defence is more sensitive to PAA in the gills than in the skin (Fig. 4). *Gpx* expression in the gills was significantly downregulated in no stress-PAA compared with the no stress-control 4 h after exposure (Fig. 4A). However, when stress status had been altered prior to PAA exposure, the level of *gpx* transcript in the gills was significantly higher in stress-PAA compared with stress-control, particularly in the early hours after exposure. The stressful episode can modulate the expression of *gpx* in salmon [35]. The elevated level of *gpx* expression in the gills of the stress-PAA group implies that stress prior to PAA treatment could increase the *gpx*-mediated antioxidant potential during oxidative stress. However, an opposite trend was an emblematic response in the expression of *gr* (Fig. 4B), *mnsod* (Fig. 4C), and *cu/znsod* (Fig. 4D) in the gills of fish subjected to stress prior to PAA exposure. The profiles revealed that gill transcription of antioxidant genes in the stress-PAA group was significantly downregulated at 1 h (i.e., *gr*, *cu/znsod*) and at 2 w (i.e., *gr*, *mnsod*, and *cu/znsod*) after exposure compared with the stress-control group. In some cases, the level of *gr* and *cu/znsod* expression in the stress-PAA group was significantly lower compared with their counterparts in the no stress-PAA group. These conspicuous transcriptional changes in the antioxidant genes within the stress group illustrate that a stressful episode prior to PAA exposure interfered with the ability of gills to mount an antioxidative response during PAA-induced oxidative stress. There were no distinct overall transcriptional changes in the skin in experiment 2 and the random changes observed were related to the temporal dynamics of gene expression (Fig. 4E–H). It appeared that the antioxidant markers were not strongly responsive to PAA, crowding stress, or their

combination in the skin.

The overall results indicate that the antioxidant defence towards PAA was more responsive in the gills than in the skin. Moreover, fish with stress history prior to PAA exposure exhibited a different mucosal antioxidative response pattern to PAA compared with the non-stressed fish, highlighting the potential confounding and compounding roles of crowding stress in the antioxidant defence. The gills have a large surface area in contact with the water and are less structurally complex than the skin, which has multiple layers [36]. Moreover, PAA and its intermediate products have a low molecular mass that might be gill-permeable and diffuse into the fish [14,15]. Therefore, this might explain, at least in part, the striking regulation of the antioxidant system in the gills relative to the skin.

### 3.4. Conclusions

Peracetic acid is a disinfectant with great promise as a prophylaxis and chemotherapeutant in aquaculture. This study shows that salmon smolts attune their systemic and mucosal defences to counteract the physiological demands of the presence of PAA, a potential oxidative stressor, in the water. PAA likely triggered systemic oxidative stress, but salmon addressed the ROS imbalance by producing circulating antioxidants. The classical stress indicators in the plasma were affected by PAA. Previous exposure history to PAA did not dramatically interfere with the stress responses, and the fish were able to recover quickly after re-exposure. Crowding stress before PAA treatment, however, did influence some of the stress indicators, particularly the level of glucose and lactate. Mucosal antioxidant defences were also affected, where changes were prominently observed in the gills. There was a clear tendency that prior stress might interfere with the mobilisation of mucosal antioxidant defences under increased ROS. The results of the present study add valuable insights into the physiological consequences of PAA exposure in salmon. The adaptive responses documented here reveal that PAA, though possibly triggering stress responses, can be used for salmon within the concentrated tested in the current study, with minimal physiological consequences, but attention must be given to confounding factors. Moreover, the data presented here have implications for the use of PAA as a routine disinfection in recirculating aquaculture system.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.08.048>.

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